

Materials and Methods

Ethical issues

All ethical guidelines were followed, with written informed consent having been obtained from the parents beforehand. No further ethical or institutional approvals were needed as patient samples and databases are included in the regular assessment of the patients. This work did not involve human or animal experiments. The provisions of the Declaration of Helsinki as revised in Tokyo 2004 do not apply to this work.

Sample collection

Genomic DNA was extracted from peripheral blood leukocytes using the salting-out protocol (Miller et al., 1988). Respiratory epithelial cells were obtained by nasal brushing, using a cytology soft sterile brush (Endobrush, Italy), in both nostrils (Rutland et al., 1982). Child was premedicated with oral paracetamol (15 mg/kg/dose) before nasal brushing. All individuals were continuously monitored for vital signs, pain complaints and/or signs of bleeding.

Sample processing for transmission electron microscopy

Nasal samples were fixed as described elsewhere (Rutland et al., 1982). Samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, Missouri, USA) in 0.1 M cacodylate buffer (Merck, Darmstadt, Germany), pH 7.2, 2h, room temperature (RT) and post-fixed with 2% osmium tetroxide, dehydrated in graded ethanol serie and treated with 1% tannic acid in 100% ethanol prior to embedding in epoxy resin (Epon). Suitable areas of ciliated cells were selected in semithin sections (1µm) and stained with methylene blue-Azure II. Ultrathin sections were cut on a LKB-ultramicrotome and retrieved on copper grids. After contrasting with aqueous uranyl acetate and lead citrate, they were observed in a JEOL 100CXII transmission electron microscope, operated at 60kV.

Cilia morphological evaluation and orientation

The ultrastructure of cilia axonemes was evaluated based on the presence of a systematic defect in any of the axonemal structures (Afzelius and Strydom, 1985). Variation in ciliary beat axis and the ciliary deviation was evaluated in a minimum of 100 transverse sections examined after printing. In printed images, a line was drawn perpendicular to the central microtubules. A reference line was then chosen based on the main orientation of the lines drawn. The angle of each line to the reference line was calculated and subtracted to the mean. These differences are near zero. The standard deviation (SD) of these differences corresponds to the variation in ciliary beat axis and ciliary deviation (De Jongh and Rutland, 1989). Measurements were conducted always by the same observer.

Whole-Exome Sequencing

Exome of the patient was sequenced using the AmpliSeq strategy on an Ion Proton next-generation sequencing (NGS) platform (Life Technologies) and variant calling was performed as described (Oliveira et al., 2015). All variants were listed in a Variant Call Format (VCF) file that was annotated and filtered using the Ion Reporter™ Software version 5.2 (<http://ionreporter.lifetechnologies.com/>) and VarAFT 2.10 (<http://varaft.eu>). As a quality control and given the technical limitations of sequencing platform (Homer, 2010), homopolymers longer than 6 bp were removed from analysis. Alamut Visual v2.10 software (Interactive Biosoftware, France) assisted variant interpretation. All the suspected variants were manually checked on the Binary Alignment Map (BAM) file through GenomeBrowse version 2.0.2 (Golden Helix, USA). Sanger sequencing was applied to validate the candidate variants, as previously reported (Pereira et al., 2015).

Copy number variation analysis

SNP-Array analysis was performed on genomic DNA of the patient using the Affymetrix1 CytoScan HD array according to the manufacturer's instructions. Arrays were scanned with the AffymetrixGeneChip1 Scanner 3000 7G and genotypes were analysed using Affymetrix Chromosome Analysis Suite Software version 3.3 (ChAS 3.3) and Annotation Net Affx Build 32.3. Interpretation was based on human reference sequence (GRCH37/hg19, Feb. 2009).

Gene expression analysis

Total RNA from nasal cell suspensions was extracted using the NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal), according to manufacturer instructions and including the optional step of "DNase treatment". The concentration and purity of RNA samples were determined on a Nanodrop spectrophotometer ND-1000 (Version 3.3; LifeTechnologies, California, USA), with the cut-off A260/A280 ratio between 1.8 to 2.1. The RNA to complementary DNA (cDNA) conversion was done with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, California, USA), according to manufacturer instructions.

Real-time quantitative PCR (qPCR) was performed to evaluate mRNA expression. qPCR was performed in a Bio-rad CFX96 (Bio-Rad, Hercules, USA) and amplifications were prepared with the NZY qPCR Green (NZYTech), in triplicate, according to manufacturer instructions. Fold variation of gene expression levels was calculated following the formula $2^{-\Delta\Delta C_t}$ (Pfaffl, 2001). The non-parametric tests used to access the expression levels differences, run in the GraphPad Prism Software (version 6.01, San Diego, USA).

Immunofluorescence Analysis

The immunofluorescence (IF) analysis of nasal epithelial cells was performed as described previously (Fliegauf et al., 2005). Briefly, cell suspensions were spread onto glass slides (STARFROST, Knittel-Glass, Germany), air dried, and stored at -80°C until use. Cells were fixed

with 4% paraformaldehyde (20 min, RT), permeabilized in 0.2% Triton X-100 (Sigma) (15 min, RT), blocked with 5% non-fat milk (60 min, RT) and incubated overnight at 4°C, with antibodies xx and mouse anti-acetylated α -tubulin (Santa Cruz Biotechnology). For each experiment, a negative control was included. Anti-rabbit IgG Alexa Fluor 488 (Invitrogen, California, USA) and anti-mouse IgG Texas Red (Santa Cruz Biotechnology Texas, USA) were used as secondary antibodies. Cells were counterstained with Vectashield mounting medium containing DAPI (Vector Laboratories). Results were observed in an epifluorescence microscope (Eclipse E400; Nikon).

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